

1 **Nematicidal Compositions and Methods of Using Them**

2

3 The present invention relates to nematicidal
4 compositions comprising a terpene component, and to
5 methods of killing nematodes by administration of a
6 nematicidal composition comprising a terpene
7 component.

8

9 Nematodes (Kingdom: *Animalia*, Phylum: *Nematoda*) are
10 microscopic round worms. They can generally be
11 described as aquatic, triploblastic, unsegmented,
12 bilaterally symmetrical roundworms, that are
13 colourless, transparent, usually bisexual, and worm-
14 shaped (vermiform), although some can become swollen
15 (pyroform). It is suggested that nematodes are the
16 most abundant form of animal life and only about 3%
17 of nematode species have been studied in detail.

18

19 Many nematodes are obligate parasites and a number
20 of species constitute a significant problem in
21 agriculture. It has been suggested that annual crop

1 loss estimates caused by plant parasitic nematodes
2 are roughly \$80 billion worldwide, with \$8 billion
3 in the USA. Nematodes are a serious pest and
4 methods to control their parasitic activities are an
5 important feature in maximising crop production in
6 modern intensive agriculture.

7
8 There are approximately 197 genera and 4300 species
9 of nematode phytoparasites. Plant parasitic
10 nematodes feed on the roots or shoots of plants.
11 The nematodes can be ectoparasites (i.e. feed on the
12 exterior of a plant) or endoparasites (i.e.
13 live/feed inside the host) and can be migratory or
14 sedentary.

15
16 Some of the most significant of the plant parasitic
17 nematodes are:

18
19 **Genus; Common name**

20 *Meloidogyne*; Root-knot nematode
21 *Pratylenchus*; Lesion nematode
22 *Heterodera*; Cyst nematode
23 *Globodera*; Cyst nematode
24 *Ditylenchus*; Stem and bulb nematode
25 *Tylenchulus*; Citrus nematode
26 *Xiphinema*; Dagger nematode
27 *Radopholus*; Burrowing nematode
28 *Rotylenchulus*; Reniform nematode
29 *Helicotylenchus*; Spiral nematode
30 *Belonolaimus*; Sting nematode
31

1 Nematodes are not just parasitic to plants but a
2 number of species are parasitic to animals, both
3 vertebrate and invertebrate. Around 50 species
4 attack humans and these include Hookworm
5 (Anclyostoma), Strongylids (Strongylus),
6 Pinworm (Enterolobius), Trichinosis (Trichina),
7 Elephantitis (Wuchereria), Heartworm (Dirofilaria),
8 and Ascarids (Ascaris).

9
10 It should be noted however that not all nematodes
11 inhabiting soil are phyto-parasitic. A number of
12 saprophagous nematodes exist which do not harm
13 plants, and indeed may actually exist in a symbiotic
14 relationship with plants.

15
16 The current procedure for the elimination of
17 nematodes in agriculture involves treating the soil
18 with methyl bromide (MB). MB essentially sterilises
19 the soil and provides effective control of a wide
20 range of soil-borne pathogens and pests, including
21 fungi, bacteria, nematodes, insects, mites, weeds
22 and parasitic plants. However, MB has a significant
23 negative impact on the environment.

24
25 Problems associated with MB include:

- 26
- 27 • Eradication of the beneficial soil microflora and
 - 28 microfauna, resulting in elimination of natural
 - 29 biological control and resurgence of secondary
 - 30 pests and diseases. The "biological vacuum"
 - 31 created by the use of potent biocides, such as

1 MB, results in rapid re-infestation of treated
2 soils.

- 3 • Toxic side-effects on humans, plants
4 (phytotoxicity) and other non-target organisms.
5 This has safety implications with regard to
6 handling MB as any contact with the user would be
7 harmful. There are therefore also major expenses
8 involved with specialist equipment, training and
9 other precautions involved with ensuring that MB
10 is used, handled and transported safely.
- 11 • MB is associated with the depletion of the ozone
12 layer.
- 13 • Pollution of the environment, including soil,
14 water and the atmosphere. MB is, in particular,
15 a major pollutant of underground water.
- 16 • Pesticide residues in agricultural products,
17 creating health risks for consumers and major
18 obstacles to the international agricultural
19 trade. Soil fumigation with MB is known to leave
20 bromine residues in the soil which can be taken
21 up by, and accumulate, in plants. Problems with
22 bromine residues in leafy vegetables, such as
23 lettuce, are quite common. Indeed, in grape
24 producing regions the use of MB is not permitted
25 due to its health implications.

26
27 For the reasons mentioned above, *inter alia*, the
28 production and use of MB is being phased out on a
29 global scale. Under the Montreal Protocol 1991, MB
30 use is to be phased out by 2005 in the E.U. and
31 other developed countries, and by 2015 in the
32 developing countries. There is therefore a need to

1 identify suitable alternative solutions for managing
2 soil-borne pathogens, in particular nematodes.

3

4 The inventor has surprisingly found that terpenes
5 are effective in killing nematodes.

6

7 Terpenes are widespread in nature, mainly in plants
8 as constituents of essential oils. Their building
9 block is the hydrocarbon isoprene (C₅H₈)_n.

10 Terpenes are classified as generally regarded as
11 safe (GRAS) by the Environmental Protection Agency
12 (EPA) in the USA and have been used in the flavour
13 and fragrance industries.

14

15 Terpenes have been found to be effective and
16 nontoxic dietary antitumor agents which act through
17 a variety of mechanisms of action (Crowell and
18 Gould, 1994 - *Crit Rev Oncog* 5(1): 1-22; and Crowell
19 *et al.*, 1996 - *Adv Exp Med Biol* 401: 131-136).

20 Terpenes, i.e. geraniol, tocotrienol, perillyl
21 alcohol, b-ionone and d-limonene, suppress hepatic
22 HMG-COA reductase activity, a rate limiting step in
23 cholesterol synthesis, and modestly lower
24 cholesterol levels in animals (Elson and Yu, 1994 -
25 *J Nutr.* 124: 607-614). D-limonene and geraniol
26 reduced mammary tumors (Elegbede *et al.*, 1984 -
27 *Carcinogenesis* 5(5): 661-664; and Elegbede *et al.*,
28 1986 - *J Natl Cancer Inst* 76(2): 323-325; and
29 Karlson *et al.*, 1996 - *Anticancer Drugs* 7(4): 422-
30 429) and suppressed the growth of transplanted
31 tumors (Yu *et al.*, 1995 - *J Agri Food Chem* 43: 2144-
32 2147).

1 Terpenes have also been found to inhibit the in-
2 vitro growth of bacteria and fungi (Chaumont and
3 Leger, 1992 - *Ann Pharm Fr* 50(3): 156-166; Moleyar
4 and Narasimham, 1992 - *Int J Food Microbiol* 16(4):
5 337-342; and Pattnaik, et al. 1997 - *Microbios*
6 89(358): 39-46) and some internal and external
7 parasites (Hooser, et al., 1986 - *J Am Vet Med Assoc*
8 189(8): 905-908). Geraniol was found to inhibit
9 growth of *Candida albicans* and *Saccharomyces*
10 *cerevisiae* strains by enhancing the rate of
11 potassium leakage and disrupting membrane fluidity
12 (Bard et al., 1988 - *Lipids* 23(6): 534-538). B-
13 ionone has antifungal activity which was determined
14 by inhibition of spore germination, and growth
15 inhibition in agar (Mikhlin et al., 1983 - *Prikl*
16 *Biokhim Mikrobiol.* 19: 795-803; and Salt et al.,
17 1986 - *Adam Physiol Molec Plant Path* 28: 287-297).
18 Teprenone (geranylgeranylacetone) has an
19 antibacterial effect on *H. pylori* (Ishii, 1993 - *Int*
20 *J Med Microbiol Virol Parasitol Infect Dis* 280(1-2):
21 239-243). Solutions of 11 different terpenes were
22 effective in inhibiting the growth of pathogenic
23 bacteria in in-vitro tests; levels ranging between
24 100 ppm and 1000 ppm were effective. The terpenes
25 were diluted in water with 1% polysorbate 20 (Kim et
26 al., 1995 - *J Agric Food Chem* 43: 2839-2845).
27 Diterpenes, i.e. trichorabdol A (from *R.*
28 *Trichocarpa*) has shown a very strong antibacterial
29 effect against *H. pylori* (Kadota et al., 1997 -
30 *Zentralblatt fur Bakteriologie.* 286(1):63-7).
31

1 Rosanol, a commercial product with 1% rose oil, has
2 been shown to inhibit the growth of several bacteria
3 (*Pseudomona*, *Staphylococcus*, *E. coli* and *H pylori*).
4 Geraniol is the active component (75%) of rose oil.

5
6 In US Patent Nos 5,977,186 and 6,130,253, methods of
7 using terpenes to kill lice are disclosed.

8
9 In International Patent Application published under
10 WO 03/020024, by the present inventor, methods of
11 using terpenes to prevent and treat infections
12 plants by bacteria, phytoplasmas, mycoplasmas or
13 fungi are disclosed.

14
15 There may be different modes of action of terpenes
16 against microorganisms; they could (1) interfere
17 with the phospholipid bilayer of the cell membrane,
18 (2) impair a variety of enzyme systems (HMG-
19 reductase), and (3) destroy or inactivate genetic
20 material. It is believed that due to the modes of
21 action of terpenes being so basic, e.g., blocking of
22 cholesterol, that infective agents will not be able
23 to build a resistance to terpenes.

24
25 There are, however, a number of drawbacks to the use
26 of terpenes. These include:

- 27 - Terpenes are liquids which can make them
28 difficult to handle and unsuitable for certain
29 purposes.
- 30 - Terpenes are not very miscible with water, and
31 it generally requires the use of detergents,
32 surfactants or other emulsifiers to prepare

1 aqueous emulsions. A stable solution can, ,
2 however, be obtained by mixing the terpenes under
3 high shear.

- 4 - Dry powder terpene formulations generally only
- 5 contain a low percentage w/w of terpenes.
- 6 - Terpenes are prone to oxidation in aqueous
- 7 emulsion systems, which make long term storage a
- 8 problem.

9
10 There are limitations to the current techniques of
11 spray coating, extrusion, coacervation, molecular
12 encapsulation, and spray drying/cooling to provide
13 ingredient delivery systems.

14
15 Yeast cell walls are derived from yeast cells and
16 are composed of the insoluble biopolymers β -1,3-
17 glucan, β -1,6-glucan, mannan and chitin. They are
18 typically 2-4 micron in diameter microspheres with a
19 shell wall that is only 0.2-0.3 micron thick
20 surrounding an open cavity. This material has
21 considerable liquid holding capacity, typically
22 absorbing 5-25 times its weight in liquid. The
23 shell is sufficiently porous that payloads up to
24 150,000 Daltons in size can pass through the outer
25 glucan shell and be absorbed into the hollow cavity
26 of the spherical particle. Yeast cell walls have
27 several unique properties, including heat stability
28 (e.g. to 121°C), shear stability, pH stability (e.g.
29 pH 2-12), and at high concentrations they do not
30 build significant viscosity. In addition to its
31 physical properties this composition contains the
32 natural and healthy dietary fibres that deliver

1 cardiovascular and immunopotential health
2 benefits.
3
4 Yeast cell walls are prepared from yeast cells by
5 the extraction and purification of the insoluble
6 particulate fraction from the soluble components of
7 the yeast cell. The fungal cell walls can be
8 produced from the insoluble byproduct of yeast
9 extract manufacture. Further, the yeast cells can
10 be treated with an aqueous hydroxide solution,
11 without disrupting the yeast cell walls, which
12 digests the protein and intracellular portion of the
13 cell, leaving the yeast cell wall component devoid
14 of significant protein contamination, and having
15 substantially the unaltered cell wall structure of
16 $\beta(1-6)$ and $\beta(1-3)$ linked glucans. A more detailed
17 description of whole glucan particles and the
18 process of preparing them is described by Jamas et
19 al. in U.S. Pat. No. 4,810,646 and in co-pending
20 patent applications U.S. Ser. No. 166,929, U.S. Ser.
21 No. 297,752 and U.S. Ser. No. 297,982. US Patent No.
22 6,242,594, assigned to Novogen Research Pty Ltd.,
23 describes a method of preparing yeast glucan
24 particles by alkali extraction, acid extraction and
25 then extraction with an organic solvent and finally
26 drying. US 5,401,727, assigned to AS Biotech-
27 Mackzymal, discloses the methods of obtaining yeast
28 glucan particles and methods of using them to
29 promote resistance in aquatic animals and as an
30 adjuvant for vaccinations. US 5,607,677, assigned
31 to Alpha-Beta Technology Inc., discloses the use of
32 hollow whole glucan particles as a delivery package

1 and adjuvant for the delivery of a variety of,
2 pharmaceutical agents. The teachings of the
3 abovementioned patents and applications are
4 incorporated herein by reference.

5
6 According to the present invention there is provided
7 a method of killing nematodes, said method
8 comprising the step of applying an effective amount
9 of a nematicidal composition comprising a terpene
10 component. Preferred features of the nematicidal
11 composition are described below.

12
13 The terpene component may comprise a single terpene
14 or a mixture of terpenes.

15
16 The list of terpenes which are exempted from US
17 regulations found in EPA regulation 40 C. F. R. Part
18 152 is incorporated herein by reference in its
19 entirety.

20
21 Preferably the terpene component comprises one or
22 more terpenes selected from the group comprising
23 citral, pinene, nerol, b-ionone, geraniol,
24 carvacrol, eugenol, carvone, terpeniol, anethole,
25 camphor, menthol, limonene, nerolidol, farnesol,
26 phytol, carotene (vitamin A), squalene, thymol,
27 tocotrienol, perillyl alcohol, borneol, myrcene,
28 simene, carene, terpenene and linalool.

29
30 It should also be noted that terpenes are also known
31 by their extract or essential oil names, e.g.
32 lemongrass oil (contains citral).

1 A suitable terpene component may comprise, for
2 example:

- 3 • 100% citral;
- 4 • 50% citral and 50% b-ionone;
- 5 • 50% citral and 50% a-terpineol;
- 6 • 50% d-limonene and 50% b-ionone; or
- 7 • 50% a-terpineol and 50% b-ionone.

8
9 It has been found that compositions comprising
10 citral are particularly effective at killing
11 nematodes. Therefore it is preferred that the
12 nematicidal composition of the present invention
13 comprises citral.

14
15 It is highly preferable that all compounds present
16 in the nematicidal composition are classified as
17 generally regarded as safe (GRAS).

18
19 The term "terpene" as used herein refers not only to
20 terpenes of formula $(C_5H_8)_n$, but also encompasses
21 terpene derivatives, such as terpene aldehydes. In
22 addition, reference to a single name of a compound
23 will encompass the various isomers of that compound.
24 For example, the term citral includes the cis-isomer
25 citral-a (or geranial) and the trans-isomer citral-b
26 (or neral).

27
28 In a preferred embodiment the nematicidal
29 composition comprises a terpene component and water.
30 The terpene component may be in solution in the
31 water. Alternatively the nematicidal composition
32 may comprise a surfactant which holds the terpene in

1 suspension in the water. Suitable surfactants
2 include, sodium lauryl sulphate, polysorbate 20,
3 polysorbate 80, polysorbate 40, polysorbate 60,
4 polyglyceryl ester, polyglyceryl monooleate,
5 decaglyceryl monocaprylate, propylene glycol
6 dicaprilate, triglycerol monostearate, TWEEN, Tween
7 80, SPAN 20, SPAN 40, SPAN 60, SPAN 80, Brig 30 or
8 mixtures thereof. Sodium lauryl sulphate is a
9 preferred surfactant due to its recognised safety.

10

11 In one embodiment of the invention the nematicidal
12 composition has a pH of less than 7, suitably a pH
13 from around 3 to less than 7, and preferably a pH
14 from around 3 to around 5. Where the nematicidal
15 composition has a pH below 7 the nematicidal
16 activity of the composition does not decrease over
17 time compared to a composition having a pH over 7.

18

19 Suitably the nematicidal composition comprises the
20 terpene component at a concentration from about 125
21 to about 2000 ppm in water, preferably from about
22 250 to about 1000 ppm. A terpene component
23 concentration from about 500 to about 2000 ppm may
24 be preferred if higher kill rates are desired.

25

26 In one embodiment of the invention the terpene
27 component is provided at a concentration at which
28 parasitic nematodes are killed selectively over non-
29 parasitic nematodes. Suitably the parasitic
30 nematodes are root-knot nematodes and the non-
31 parasitic nematodes are Saprophagous nematodes.

1 Suitable concentrations include from 250 to 1000
2 ppm, and preferably from 250 to 750 ppm.

3
4 The nematicidal composition may also comprise an
5 excipient. The excipient may suitably comprise a
6 liposome. Certain excipients may augment the action
7 of the terpene component by, for example, increasing
8 its longevity of action or by increasing its
9 capacity to contact and interact with nematodes.

10
11 A particularly preferred excipient is hollow glucan
12 particles. The term "hollow glucan particle" as
13 used herein includes any hollow particle comprising
14 glucan as a structural component. Thus, in
15 particular, the term includes yeast cell walls (in
16 purified or crude forms) or other hollow glucan
17 particles, which may be hollow whole glucan
18 particles.

19
20 It has been found that terpenes can be taken up and
21 stably encapsulated within hollow glucan particles.

22
23 According to a further aspect of the present
24 invention there is provided a method of killing
25 nematodes, said method comprising the step of
26 applying an effective amount of a nematicidal
27 composition comprising a hollow glucan particle
28 encapsulating a terpene component.

29
30 Nematicidal compositions comprising a hollow glucan
31 particle encapsulating a terpene component can
32 provide the following advantages:

- 1 - maximise terpene payload;
- 2 - minimise unencapsulated payload;
- 3 - control payload stability;
- 4 - control payload release kinetics;
- 5 - creation of a solid form of a liquid terpene to
- 6 increase the mass and uniformity;
- 7 - simplify handling and application of terpenes;
- 8 and
- 9 - mask the smell and taste of the terpene.

10

11 Preferably the hollow glucan particles are yeast
12 cell walls. Yeast cell walls are preparations of
13 yeast cells that retain the three-dimensional
14 structure of the yeast cell from which they are
15 derived. Thus they have a hollow structure which
16 allows the terpene component to be encapsulated
17 within the yeast cell walls. The yeast walls may
18 suitably be derived from Baker's yeast cells
19 (available from Sigma Chemical Corp., St. Louis,
20 MO).

21

22 Alternative particles are those known by the trade
23 names SAF-Mannan (SAF Agri, Minneapolis, MN) and
24 Nutrex (Sensient Technologies, Milwaukee, WI).
25 These are hollow glucan particles that are the
26 insoluble waste stream from the yeast extract
27 manufacturing process. During the production of
28 yeast extracts the soluble components of partially
29 autolyzed yeast cells are removed and the insoluble
30 residue is a suitable material for terpene loading.
31 These hollow glucan particles are ~25-35% glucan
32 w/w. A key attribute of these materials are that

1 they are >10% lipid w/w and are very effective at
2 absorbing terpenes. In addition, as a waste stream
3 product they are a relatively cheap cost source of
4 hollow glucan particles.

5
6 Alternative hollow glucan particles which have
7 higher purity are those produced by Nutricepts
8 (Nutraceuticals Inc., Burnsville, MN) and ASA Biotech.
9 These particles have been alkali extracted, which
10 removes additional intracellular components as well
11 as removes the outer mannoprotein layer of the cell
12 wall yielding a particle of 50-65% glucan w/w.
13 Since alkali extraction saponifies some of the
14 lipids these particles are less effective at
15 absorbing terpenes. They are also significantly
16 more expensive and hence these materials are
17 preferred particles.

18
19 Higher purity hollow glucan particles are the WGP
20 particles from Biopolymer Engineering. These
21 particles are acid extracted removing additional
22 yeast components yielding a product 75-85% glucan
23 w/w. They are even more expensive than the
24 Nutricepts and ASA Biotech particles and the lower
25 lipid content results in poor loading with terpenes.

26
27 Very high purity hollow glucan particles are WGP
28 from Alpha-beta Technology, Inc. (Worcester, MA) and
29 microparticulate glucan from Novogen (Stamford, CT).
30 These particles are organic solvent extracted
31 removing residual lipids and are >90% glucan w/w.

1 Of all of the materials tested so far, these ,
2 particles absorbed the least terpenes.

3

4 Situations may, however, be envisaged where a high
5 purity glucan particle is required, for example,
6 where tight control over possible contaminants is
7 required. In these instances the higher purity
8 particles would be preferred over the more crude
9 products, despite their poorer terpene loading
10 characteristics.

11

12 Preferably the hollow glucan particles have a slight
13 lipid content. A slight lipid content can increase
14 the ability of the hollow glucan particle to
15 encapsulate the terpene component. Preferably the
16 lipid content of the hollow glucan particles is
17 greater than 5% w/w, more preferably greater than
18 10% w/w.

19

20 For encapsulation into a hollow glucan particle the
21 terpene component of the present invention can
22 optionally be associated with a surfactant. The
23 surfactant can be non-ionic, cationic, or anionic.
24 Examples of suitable surfactants include sodium
25 lauryl sulphate, polysorbate 20, polysorbate 80,
26 polysorbate 40, polysorbate 60, polyglyceryl ester,
27 polyglyceryl monooleate, decaglyceryl monocaprylate,
28 propylene glycol dicaprylate, triglycerol
29 monostearate, Tween®, Tween 80, Span® 20, Span® 40,
30 Span® 60, Span® 80, Brig 30 or mixtures thereof.
31 The surfactant acts to hold the terpene component in

1 an emulsion and also assists encapsulation of the
2 terpene component into the hollow glucan particle.

3
4 The nematicidal composition of the invention can
5 comprise hollow glucan particles encapsulating a
6 terpene component which comprise 1 to 99% by volume
7 terpene component, 0 to 99% by volume surfactant and
8 1 to 99% hollow glucan particles. More specifically
9 the hollow glucan particles encapsulating a terpene
10 component can comprise from about 10% to about 67%
11 w/w terpene component, about 0.1-10% surfactant and
12 about 40-90% hollow glucan particles. A stable
13 suspension of hollow glucan particles incorporating
14 citral of 25 ppt citral can be made.

15
16 Suitably a nematicidal composition comprises from
17 about 500 to about 10,000 ppm hollow glucan
18 particles, where the particles contain from about 1
19 to about 67 % terpene component. Preferably the
20 nematicidal composition comprises from about 1000 to
21 about 2000 ppm hollow glucan particles, where the
22 particles contain from about 10 to about 50% terpene
23 component.

24
25 The method is particularly suited to killing
26 nematodes in soil, especially in soil used for
27 agricultural or horticultural purposes. Such a
28 method involves administering a nematicidal
29 composition comprising a terpene component to at
30 least a portion of, preferably all of, the soil to
31 be treated.

32

1 Optionally the application of the nematicidal,
2 composition may be repeated. This may be necessary
3 in some cases to ensure effective killing of the
4 nematodes present in the portion of soil.
5 The application of the nematicidal composition to
6 soil may be carried out in a number of ways,
7 including spraying, irrigation or the like.

8
9 In one embodiment the nematicidal composition used
10 in the method of the present invention may be formed
11 by mixing the terpene component and water with
12 sufficient shear to create a solution of the terpene
13 in water. Terpenes are generally poorly soluble in
14 water, however, with mixing at sufficient shear they
15 can be forced to form a stable solution in water.
16 An aqueous terpene solution has the advantage that
17 it can be taken up by plants through their roots,
18 whereas an aqueous terpene suspension cannot.

19
20 In an alternative embodiment the nematicidal
21 composition may be formed by adding a surfactant to
22 hold the terpene component in aqueous suspension.
23 Such a suspension would be useful where it is not
24 necessary for the composition to be taken up by the
25 plant, e.g. for treating an infection with
26 ectoparasitic nematodes.

27
28 In an alternative embodiment the present invention
29 further provides a method of preparing a nematicidal
30 composition comprising hollow glucan particles
31 encapsulating a terpene component, said method
32 comprising the steps of;

- 1 a) providing a terpene component;
- 2 b) providing hollow glucan particles;
- 3 c) incubating the terpene component with the
- 4 glucan particles under suitable conditions
- 5 for terpene encapsulation; and
- 6 d) recovering the glucan particles
- 7 encapsulating the terpene component.

8

9 Optionally the above method can further comprise the

10 step of drying the glucan particles encapsulating

11 the terpene component. Drying may be achieved in a

12 number of ways and mention may be made of freeze

13 drying, fluidised bed drying, drum drying or spray

14 drying, all of which are well known processes.

15

16 In step a) of the above method, the terpene

17 component is suitably provided as a suspension in an

18 aqueous solvent, and optionally in the presence of a

19 surfactant. Suitably the solvent is water. A

20 suitable surfactant is Tween-80

21 (polyoxyethylenesorbitan monooleate) or sodium

22 lauryl sulphate, and preferably the surfactant is

23 present at a concentration of about 0.1 to 10 % by

24 volume of the total reaction mixture, more

25 preferably about 1%. Alternatively the terpene

26 component may be provided as a true solution in a

27 solvent, e.g. water. A true solution of terpene in

28 water can be obtained by mixing the terpene in water

29 at high shear until a true solution is obtained.

30 Publication No WO03/020024 provides further details

31 of forming true solutions of terpenes in water.

32

1 In step b) of the above method, the hollow glucan
2 particles are suitably provided as a suspension in
3 water or other suitable liquid. Suitably the
4 suspension comprises approximately 1 to 1000 mg
5 glucan particles per ml, preferably 200 to 400
6 mg/ml. Alternatively the hollow glucan particles
7 may be provided as a dry powder and added to the
8 terpene-surfactant suspension.

9
10 Alternatively the glucan particles are provided in
11 sufficient liquid to minimally hydrate the
12 particles, but not in significant excess. The term
13 "hydrodynamic volume" (HV) is used to describe the
14 volume of liquid required to minimally hydrate the
15 particles. Thus suitably the particles are provided
16 in between the HV and HV + 50% of water. This makes
17 the subsequent drying step more efficient. Also,
18 where a low volume of water is used (ie. around HV
19 to HV + 50%), it is also possible to extrude the
20 finished product into pellet or noodle form, which
21 is convenient for fluidised bed drying.

22
23 It has been found that the terpene component can
24 become encapsulated by the hollow glucan particles
25 at room temperature. The rate of encapsulation is,
26 however, increased at 37°C but the temperature
27 should be kept below the boiling point or denaturing
28 temperature of any component of the composition.
29 Suitable conditions for step c) of the above method
30 are therefore atmospheric pressure at a temperature
31 of 20 to 37°C. Optimisation of the conditions for a

1 particular encapsulation reaction will be a matter
2 of routine experimentation.

3
4 The present invention also provides the use of a
5 nematicidal composition comprising a terpene
6 component as described above for the extermination
7 of nematodes, especially nematodes in soils and/or
8 infecting plants.

9
10 It will be obvious to one skilled in the art that
11 the nematicidal use of a composition made entirely
12 of compounds which are GRAS is highly preferable
13 over the use of prior art toxic compositions.
14 Environmental concerns associated with use of the
15 composition will be greatly reduced and there would
16 be no significant problems with accumulation of the
17 product in food crops. Additionally, regulatory
18 approval of the composition in various jurisdictions
19 would not be as difficult to obtain as for a toxic
20 composition, and indeed may not even be required in
21 some instances.

22
23 Embodiments of the present invention will now be
24 described by way of example only, with reference to
25 the figures in which:

26
27 Fig. 1 represents a light micrograph of empty yeast
28 cell walls;

29 Fig. 2 represents a light micrograph of yeast cell
30 walls encapsulating L-carvone;

31 Fig. 3 represents a light micrograph of yeast cell
32 walls encapsulating citral;

1 Fig. 4 represents a light micrograph of terpene
2 emulsion;
3 Fig. 5 represents a light micrograph of yeast cell
4 walls in hydrodynamic volume (HV) water;
5 Fig. 6 represents a light micrograph of yeast cell
6 walls encapsulating terpene in 5 times hydrodynamic
7 volume (HV) of water;
8 Fig. 7 represents a light micrograph of yeast cell
9 walls encapsulating terpene in HV of water;
10 Fig. 8 represents a light micrograph of yeast cell
11 walls encapsulating terpene in HV plus 5% of water;
12 Fig. 9 represents a light micrograph of yeast cell
13 walls encapsulating terpene in HV plus 10% of water;
14 Fig. 10 represents a light micrograph of yeast cell
15 walls encapsulating terpene in HV plus 20% of water;
16 Fig. 11 represents a light micrograph of yeast cell
17 walls encapsulating terpene in HV plus 30% of water;
18 Fig. 12 represents a light micrograph of yeast cell
19 walls encapsulating terpene in HV plus 40% of water.
20 Fig. 13 represents a light micrograph showing the
21 dispersal of dried hollow glucan particles
22 encapsulating a terpene component and no xanthan
23 gum.
24 Fig. 14 represents a light micrograph as in Fig. 13
25 where 0.07 g of 1% xanthan gum is included.
26 Fig. 15 represents a light micrograph as in Fig. 13
27 where 0.14 g of 1% xanthan gum is included.
28 Fig. 16 represents a light micrograph as in Fig. 13
29 where 0.28 g of 1% xanthan gum is included.
30 Fig. 17 represents a light micrograph as in Fig. 13
31 where 0.55 g of 1% xanthan gum is included.

1 Fig. 18 represents a light micrograph as in Fig. 13
2 where 1.1 g of 1% xanthan gum is included.
3 Fig. 19 represents a light micrograph as in Fig. 13
4 where 2.2 g of 1% xanthan gum is included.
5 Fig. 20 represents a light micrograph as in Fig. 13
6 where 4.4 g of 1% xanthan gum is included.

7

8 Example 1 - Preparation of a terpene emulsion or
9 suspension using a surfactant

10

11 A terpene, terpene mixture, or liposome-terpene
12 combination can be combined with a surfactant to
13 form a suspension. The volumetric ratio of terpenes
14 is generally about 1-99%, and the surfactant
15 volumetric ratio is about 1-50% of the
16 solution/mixture. The terpenes, comprised of
17 natural or synthetic terpenes, are added to water.
18 The surfactant, preferably polysorbate 80 or other
19 suitable GRAS surfactant, is added to the
20 water/terpene mixture and then blended to form a
21 suspension. Citral is a suitable terpene.

22

23 Example 2 - Preparation of a terpene solution
24 (without surfactant)

25

26 The solution can be prepared without a surfactant by
27 placing the terpene, e. g. citral, in water and
28 mixing under solution-forming shear conditions until
29 the terpene is in solution.

30

31 0.5 ml citral was added to 1 litre water. The
32 citral and water were blended in a household blender

1 for 30 seconds.

2

3 Alternatively, moderate agitation also prepared a
4 solution of citral by shaking by hand for
5 approximately 2-3 minutes.

6

7 Greater than about zero ppm to about 1000 ppm of
8 natural or synthetic terpenes such as citral, b-
9 ionone, geraniol, carvone, terpeniol, carvacrol,
10 anethole, or other terpenes with similar properties
11 are added to water and subjected to a solution-
12 forming shear blending action that forces the
13 terpene(s) into a true solution. The maximum level
14 of terpene(s) that can be solubilized varies with
15 each terpene. Examples of these levels are shown in
16 Table 1.

17

18 **Table 1** - Solution levels for various terpenes.

19

<u>Terpene</u>	<u>Solution Level</u>
Citral	1000 ppm
Terpeniol	500 ppm
b-ionone	500 ppm
Geraniol	500 ppm
Carvone	500 ppm

20

21 Example 3 - Potency of solution

22

23 Terpenes will break down in the presence of oxygen.
24 The rate at which they decay varies for each
25 particular terpene.

26

1 Citral is a terpene aldehyde and will decay over a
2 period of days. Two protocols are described below
3 which quantify the rate of decay of citral.

4
5 The following protocol was used to determine the
6 rate of decay of citral in a sealed container:

7

8 **Test Material**

9 A solution prepared as described in Example 2
10 containing citral at 1000 ppm was prepared in
11 distilled water. This solution was stored in a
12 capped glass vial for the duration of the test.

13

14 **Procedure**

15 A standard curve was prepared with citral and B-
16 ionone as internal standard.

17

18 At the beginning of the study and weekly for four
19 weeks the 1000 ppm suspension was analyzed using a
20 gas chromatography procedure. The concentration of
21 citral was determined by plotting it on the standard
22 curve.

23

24 The results are shown below in Table 2.

25

26 **Table 2 - Stability of citral**

27

	Percentage of citral remaining			
	Day 1	Week 1	Week 2	Week 4
Citral (1000 ppm)	100	32	27	22

28

1 The following protocol was used to determine the
2 rate of decay of citral in a container with a porous
3 lid.

4

5 To determine the concentration of citral in water
6 the following protocol was used.

7

8 **Test Material**

9 A solution containing citral at 1000 ppm was
10 prepared in distilled water. This solution was
11 stored in a beaker covered with porous paper for the
12 duration of the test.

13

14 **Procedure**

15 A standard curve was prepared with citral and B-
16 ionone as internal standard.

17

18 At the beginning of the study and after a week the
19 1000 ppm suspension was analyzed using a gas
20 chromatography procedure. The concentration of
21 citral was determined by plotting it on the standard
22 curve.

23

24 The results are shown below in Table 3.

25

26 **Table 3 - Stability of citral**

27

	Percentage of citral remaining	
	Day 1	Week 1
Citral (1000 ppm)	100	21.5%

28

1 Example 4 - Extraction of Nematode Eggs from Soil
2 and counting nematode numbers

3
4 **Extraction of Eggs and Quantification of Soil**
5 **Populations**

6
7 The following is an outline of a suitable technique
8 to determine the population densities of soybean
9 cyst nematodes SCN in soil samples, although it
10 would be applicable to other soil nematodes. The
11 procedure has three stages:

- 12 • extracting the cysts from the soil;
- 13 • crushing the cysts to extract the eggs; and,
- 14 • microscopic observation of the suspension of
- 15 eggs for counting.

16
17 **Extraction of cysts from soil**

18
19 Cysts of soybean cyst nematode are recovered from
20 soil through a combination of wet-sieving and
21 decanting. The technique is a modification of the
22 Cobb (Cobb, N.A. 1918. Estimating the nema
23 population of soil. U.S. Dept. Agr. Bur. Plant Ind.
24 Agr. Tech. Cir., 1:1-48) sifting and gravity
25 technique.

26
27 The procedure is as follows:

- 28 1. Combine a well mixed 100 cm³ soil sample (approx.
29 1/2 cup) in a bucket with two (2) quarts (2.27
30 litres) of water.
- 31 2. Break any clumps with your fingers and mix the
32 soil suspension well for 15 seconds.

- 1 3. Pour the soil suspension through an 8-inch-,
2 diameter #20 (850 mm pore) sieve into another
3 bucket. Briefly rinse the debris caught on the 20
4 mesh sieve.
- 5 4. Pour the soil suspension in the second bucket
6 through a #60 (250 mm pore) sieve.
- 7 5. Backwash the debris caught on the 60 mesh screen
8 into a pan.
- 9 6. Repour the suspension through the 60 mesh screen
10 - hold the screen at an angle to concentrate the
11 cysts and debris.
- 12 7. Backwash into a pan using a minimal (<250 ml)
13 amount of water.
- 14 8. Pour the cysts and debris into a 250 ml beaker.
- 15 **NOTE:** Discard the heavier material that quickly
16 settles to the bottom of the buckets/pans during
17 the above sieving process.

18 19 **Extraction of eggs from the cysts**

20
21 The above technique will result in a suspension of
22 SCN cysts, along with organic debris and sediments
23 similar in size to the cysts. The cysts in this
24 suspension could be counted using a simple
25 dissecting microscope. Some laboratories that
26 analyze soil for soybean cyst nematode report
27 results in the form of cysts per 100 cm³ of soil.
28 Egg content of cysts is highly variable, and will
29 not yield reliable counts of the SCN population in
30 the sample. Therefore, it is preferable if eggs are
31 extracted from the cysts and results are reported

1 back as eggs and second stage juveniles (J-2) per
2 100 cm³ of soil.

3

4 The procedure used to extract eggs from cysts is as
5 follows:

- 6 1. Allow cysts/debris to settle for ca 30 minutes
7 in the 250 ml beakers. Pour off excess water,
8 resuspend sediments and transfer to 50 ml
9 beakers.
- 10 2. Allow cysts to settle in the 50 ml beakers.
- 11 3. Pour off excess water (~30 ml) and transfer the
12 cyst/debris suspension to a 55 ml Wheaton
13 Potter-Elvehjen tissue grinder.
- 14 4. Grind at 7500 RPM for 10 seconds. Rinse pestle
15 into grinding tube.
- 16 5. After grinding, pour the suspension in the tube
17 through an 8-inch-diameter #200 (75 mm pore)
18 sieve over a stainless steel #500 (25 mm pore)
19 sieve.
- 20 6. Rinse the tube several times with tap water,
21 each time pouring the contents through the
22 sieves. Discard sediments caught on the #200
23 sieve.
- 24 7. Carefully wash sediments and eggs caught on the
25 #500 sieve into a clean beaker with as little
26 water as possible.

27

28 **Counting eggs with the nematode counting slide:**

29

30 The volume of the egg suspension should be brought
31 up to exactly 50 ml with tap water. Fill the
32 chamber of the nematode counting slide with a well-

1 mixed suspension using a pipette. The specially
2 made nematode counting slides are constructed so
3 that the volume of egg suspension observed over the
4 grid is exactly 1 ml. Consequently, simply count
5 the number of eggs that appear within the grid of
6 the slide to determine the number of eggs per ml of
7 suspension. The total number of eggs in the sample
8 can then be calculated by multiplying the number of
9 eggs per ml by 50.

10

11 Sources of materials and equipment

12

13 Sieves:

- 14 • Fisher Scientific, 1600 W. Glenlake Avenue,
15 Itasca, IL 60143 - (800) 223-9114
- 16 • VWR Scientific, P.O. Box 66929, O'Hare AMF,
17 Chicago, IL 60666 - (800) 932-5000

18

19 Tissue Grinder:

- 20 • Fisher Scientific, 1600 W. Glenlake Avenue,
21 Itasca, IL 60143 - (800) 223-9114

22

23 Motorized stirrer:

24 The motorized laboratory stirrer is a Talboys Model
25 101 stirrer. This stirrer can be purchased through
26 VWR Scientific or directly through Talboys
27 Engineering Corporation, South Montrose, PA 18843.

28

29 Nematode counting slides:

30

31 The specially made nematode counting slides can be
32 purchased from Advanced Equine Products, 5004 228th

1 Avenue S.E., Issaquah, Washington 98029, (425) 391-
2 1169, FAX (425) 391-6669.

3

4 Example 5 - Effect of Terpenes on Nematode Egg
5 Hatching and Juvenile Survival

6

7 The effect of various terpene containing
8 compositions was assessed in relation to nematode
9 eggs and juvenile nematodes.

10

11 The protocol used was as follows:

12

13 The live eggs were treated in the various
14 samples for one hour, rinsed, put back into
15 distilled water and counted 24 hours later. The
16 samples were made up as shown in Table 4a:

17

18 **Table 4a**

19

Sample	Components		
NM1	10% Tween 80	45% d-limonene	45% b-Ionone
NM3	10% Tween 80	45% citral	45% b-Ionone
NM5	10% Tween 80	45% citral	45% a-terpineol
NM6	10% Brig 30	45% a-terpineol	45% b-Ionone
NM7	10% Tween 80	45% a-terpineol	45% b-Ionone

20

21

22

23

24

25

1 The results of the protocol are shown below in Table
2 4b.

3

4 **Table 4b - Results**

5

Sample Designation	Conc. (%)	Egg batch (%)	Juveniles alive (%)
Control	---	19	86
NM1	0.5	3	0
	0.1	10	19
	0.05	17	67
NM3	0.5	2	1
	0.1	5	3
	0.05	10	31
NM5	0.5	4	0
	0.1	9	16
	0.05	16	37
NM6	0.5	11	13
	0.1	17	36
	0.05	16	48
N6	0.5	26	53
	0.1	26	58
	0.05	15	60
NM7	0.5	13	74
	0.1	13	58
	0.05	17	75

6

7 Observations: The combinations containing citral
8 (NM3 and NM5) were more effective. The Brig
9 surfactant was not as effective as Tween 80. The
10 aldehyde worked better than the alcohols.

11

12

13

1 Example 6 - Effect of Terpenes on Mature Root-Knot,
2 Ring and Citrus Nematodes

3

4 The effect of various terpene containing
5 compositions was assessed in relation to Root-Knot
6 nematodes (*Meloidogyne*), Ring nematodes
7 (*Criconemella xenoplax*) and Citrus nematodes
8 (*Tylenchulus semipenetrans*).

9

10 The protocol used was as follows:

11

12 Nematodes: A single 5 ml volume with pre-counted
13 nematode numbers was used as the initial inoculum.
14 Nematodes were collected, identified and maintained
15 from commercial agricultural crops soils. The
16 nematodes were counted and evaluated for good health
17 for the duration of the study.

18

19 Nematicidal compositions: In this protocol the
20 terpene used in the nematicidal composition was
21 citral. The relevant details of the citral used are
22 as follows:

23

24	Chemical Name:	CITRAL
25	Common Name:	Lemongrass Oil
26	Formulation:	CITRAL FCC
27	Product Trade Name:	CITRAL FCC
28	Product code:	03-29200
29	Source:	Penta Manufacturing
30	Lot Numbers:	77887
31	Type:	Liquid
32	Carrier:	Distilled Water

1 Storage Conditions: Ambient indoor room temperature
2 ~65°F (28.3°C).

3 Stability: Insoluble in water above 1,000
4 ppm.

5
6 3 different concentrations of citral were used to
7 assess the efficacy of terpenes in killing the
8 nematodes. These were untreated control (UTC),
9 500ppm and maximum soluble terpene concentration
10 (900ppm). The terpenes were combined with water as
11 a solution by mixing at a solution forming shear.
12 The 900 ppm concentration value was not be measured,
13 but estimated at the maximum soluble concentration
14 that can be obtained with distilled water at 65°F
15 (28.3°C). 3 replicates of the 900ppm concentration
16 were used (R1, R2 and R3) and one replicate of the
17 500 ppm concentration and UTC.

18
19 Test mixtures of nematodes and the nematicidal
20 compositions were made up according to Table 5.

21
22 **Table 5 - Test Mixtures**

23

Label	Nematode Vol. ml	Terpene Conc. ppm	Added Terpene Vol. Ml	Nematode + Terpene Vol.	Treatment Conc. ppm
UTC	5.0	0.0	5.0	10.0	0.0
1.0	5.0	500.0	15.0	20.0	375.0
R1	5.0	900.0	15.0	20.0	675.0
R2	5.0	900.0	15.0	20.0	675.0
R3	5.0	900.0	15.0	20.0	675.0

24

1 The terpene and nematode containing water was
2 combined to form a final dilution volume and
3 maintained in vials between evaluations. The
4 nematodes were exposed to the terpenes for between
5 48 to 72 hours depending on their survival.

6
7 Evaluations: Nematodes were be counted and their
8 appearance assessed by microscope. The microscope
9 used for assay provided for only 5 ml to be viewed
10 at one time. Therefore, the 20 ml of total terpene
11 nematode sample water was divided into 4 parts for
12 each assay and recombined afterwards. The rating of
13 degree of efficacy of the test samples was
14 determined by observing nematode mobility,
15 mortality, and internal disruption or vacuolation
16 over time.

17
18 The results are shown below in Table 6.

19
20
21
22
23
24
25
26
27
28
29
30
31
32

1 **Table 6 - Results**

2

Sample I.D.			Root-Knot		Ring		Citrus	
(pretreatment reading)			Meloidogyne		CX		TS	
Day	Treatment	Time	Alive	Dead	alive	Dead	alive	dead
1	UTC	11:00am	351	0	357	0	148	0
1	1.0	11:00am	359	0	325	0	119	0
1	20ml-R1	11:00am	326	0	264	0	132	0
1	20ml-R2	11:00am	347	0	260	0	141	0
1	20ml-R3	11:00am	328	0	442	0	137	0
(posttreatment readings)								
1	UTC	6:00pm	348	0	350	0	144	0
1	1.0	6:00pm	355	0	319	0	114	0
1	20ml-R1	6:00pm	320	0	258	0	128	0
1	20ml-R2	6:00pm	341	0	255	0	139	0
1	20ml-R3	6:00pm	325	0	436	0	134	0
2	UTC	6:00am	344	0	348	0	140	0
2	1.0	6:00am	350	0	312	0	112	0
2	20ml-R1	6:00am	140	176	91	0	160	0
2	20ml-R2	6:00am	168	169	110	141	46	84
2	20ml-R3	6:00am	137	184	181	248	70	59
2	UTC	6:00am	340	0	342	0	135	0
2	1.0	6:00am	340	6	304	4	101	8
2	20ml-R1	6:00am	0	302	0	239	0	109
2	20ml-R2	6:00am	0	322	0	236	0	116
2	20ml-R3	6:00am	0	305	0	402	0	117
3	UTC	6:00am	330	3	336	1	126	5
3	1.0	6:00am	189	149	190	108	47	51

3

1 There was a small nematode loss from one reading to
2 another due to nematodes hanging up on the sides of
3 dishes and vials. These populations are usually
4 under 5 nematodes per reading.

5

6 Observations:

7

8 Day 1 - pretreatment readings showed no dead
9 nematodes and the nematodes were all moving and had
10 no internal disruption or vacuolation.

11

12 Day 1 - 6pm (20ml - R1+R2+R3) treatments all
13 appeared to have slowed movement but they had no
14 internal disruption or vacuolation.

15

16 Day 1 - 6pm (1.0 and UTC) treatments showed no
17 slowing of movement or internal disruption or
18 vacuolation.

19

20 Day 2 - 6am (UTC and 1.0) treatments all appeared
21 normal with no loss of movement and no internal
22 disruption or vacuolation.

23

24 Day 2 - 6am (20ml - R1+R2+R3) treatments had some
25 dead (dead had no movement and their internal body
26 structures were highly vacuolated). The living
27 nematodes were still moving, although slowly, but no
28 internal disruption or vacuolation.

29

30 Day 2 - 6pm (UTC) treatment all appeared normal with
31 no loss of movement and not internal disruption or
32 vacuolation.

1 . Day 2 - 6pm (1.0) treatment had some dead. Dead had
2 no movement with internal disruption and
3 vacuolation. Some of the living had slowed movement
4 and some did not, but none had any internal
5 disruption or vacuolation.

6

7 Day 2 - 6pm (20ml - R1+R2+R3) treatments were all
8 dead with no movement and internal disruption with
9 vacuolation.

10

11 Day 3 - 6am (UTC) treatments showed a few dead or
12 dyeing nematodes. They had no movement but showed
13 no internal disruption or vacuolation. The rest of
14 the nematodes, listed as alive, still had good
15 movement.

16

17 Day 3 - 6am (1.0) treatments showed about 50% dead
18 and both internal disruption and vacuolation. The
19 alive nematodes showed some slowing of movement but
20 no internal disruption or vacuolation.

21

22 As can be clearly seen from the results, on day two
23 by 6pm, compositions R1, R2 and R3 had killed all
24 nematodes. This demonstrates the highly nematicidal
25 properties of compositions R1, R2 and R3 and
26 consequently the nematicidal properties of citral.

27

28 Example 7 - Effect of Citral Alone and Citral and
29 Thymol on Root-Knot Nematode Juveniles

30

31 Treatment samples were prepared as follows:

32

1 Citral - 1 ml citral was added to 400ml of sterile
2 distilled water and mixed using a household blender
3 for 40 seconds. This was labelled 2500 ppm and was
4 diluted to provide test solutions at 500, 250, 125
5 and 62.5 ppm.

6
7 Citral and Thymol - 1.0 g of thymol was dissolved in
8 1 ml of citral and blended in 400 ml of water as for
9 citral alone. This was marked 2500 ppm and diluted
10 to provide test solutions at 500, 250, 125 and 62.5
11 ppm.

12
13 Control - Water was used as the control.

14
15 Nematode juveniles were collected in water and 0.1
16 to 0.15 ml added to each well of a plastic assay
17 plate. 1.0 ml of the test solutions was added to
18 each well. Observations were made microscopically
19 after 24 and 48 hours as described in Example 4.
20 Dead nematodes adopt a straight position and do not
21 move when probed with a fine needle. Living
22 nematodes move in an undulating, wave-like motion.

23
24 The results of two experiments are provided below in
25 Tables 7 and 8. The figures given are for the
26 percentage of nematodes found to be dead upon
27 microscopic examination and are the average of 2.
28 replicates.

29
30 **Table 7** - Effect of test solutions of root-knot
31 juveniles after 24 and 48 hours .

32

	Citral and Thymol			Cital (ppm)			Control
Test (ppm)	500	250	125	500	250	125	Water
24h	100	100	100	98	100	100	10
48h	100	91	50	97	91	24	31

1

2 **Table 8** - Effect of test solutions of root-knot
3 juveniles after 24 hours.

4

	Citral and Thymol			Cital (ppm)			Control
Test (ppm)	250	125	62.5	250	125	62.5	Water
24h	97	96	94	94	94	98	6

5

6 The results demonstrate the ability of citral alone
7 and a citral and thymol mixture to kill nematodes at
8 low concentrations. Kill rates in table 7 after 48
9 hours were over 90% for both mixtures at 250 ppm and
10 500 ppm concentrations. The 125 ppm concentration
11 showed a lower kill rate. The kill rates in Table 8
12 show high kill rates after 24 hours for
13 concentration as low as 62.5 ppm.

14

15 The mixture of thymol and citral did not show a
16 significant increase in kill rate over citral alone.

17

18 The results show that citral is an effective
19 nematicide even at low concentrations.

20

21

22

1 Example 8 - Effects of Citral on Root-Knot Nematodes
2 vs Saprophagous Nematodes

3
4 The purpose of this experiment was to demonstrate
5 that citral selectively kills the harmful root-knot
6 nematodes over saprophagus nematodes, which are not
7 harmful, and indeed may be beneficial to the plant
8 and soil. Such selective killing is a surprising
9 effect that means treatment with terpenes may kill
10 parasitic nematodes, but not eliminate the
11 beneficial micro-fauna in the soil.

12
13 Aqueous text mixtures comprising 250 ppm citral
14 alone and 250 ppm citral and 10% tween were produced
15 according to the techniques described in Example 7
16 above. These compositions were then incubated with
17 root-knot and saprophagus nematodes and the kill
18 rate assessed microscopically. Living saprophagus
19 nematodes move rapidly in water. The control used
20 was the nematodes in water alone.

21
22 The results are provided in Tables 9 and 10 below.
23 The figures given are for the percentage of
24 nematodes found to be dead upon microscopic
25 examination and are the average of 2 replicates.

26
27
28
29
30
31

1 **Table 9-** Nematicidal activity of citral on root-knot
2 nematodes (% dead)

3

	Citral +Tween 80 (250 ppm)	Citral (250 ppm)	Citral +Tween 80 (250 ppm)	Citral (250 ppm)	Control
24 h	87	87	89	88	17
48h	100	100	100	100	22

4
5 **Table 10-** Nematicidal activity of citral on
6 Saprophagous nematodes (% dead)

7

	Citral +Tween 80 (250 ppm)	Citral (250 ppm)	Citral +Tween 80 (250 ppm)	Citral (250 ppm)	Control
24h	45	43	51	50	15
48h	50	50	53	52	19

8
9 The results clearly show that citral kills the
10 pathogenic root-knot nematodes at a much higher kill
11 rate than the beneficial saprophagus nematodes.
12 After 48hrs the kill rate for root-knot nematodes
13 was 100% for all test mixtures, whereas for
14 Saprophagus nematodes it was only 50-53%. The
15 results were not significantly effected by the
16 inclusion of Tween 80.

17
18 The results demonstrate that terpenes have the
19 ability to selectively kill pathogenic nematodes
20 whilst allowing beneficial nematodes to survive in
21 the soil. This would result in a more healthy soil
22 environment post treatment than a treatment which

1 kills the entire nematode population in the soil.
2 Firstly this is because beneficial nematodes would
3 be present in the soil post treatment, and secondly
4 there would not be a nematode "vacuum" in the soil
5 which could be filled with pathogenic nematodes or
6 other pathogens.

7
8 It could be expected that at a very high
9 concentration of terpene may result in a higher kill
10 rate of saprophagus nematodes, thus reducing the
11 selectivity of the treatment. Therefore in use in
12 the field the minimum concentration that achieves
13 the desired kill rate in root-knot or other
14 parasitic nematodes may be selected, thus maximising
15 the selectivity.

16
17 Example 9 - Effect of pH on the Nematicidal Activity
18 of Citral containing compositions.

19
20 The following protocol was performed to assess the
21 affect of pH on test solutions containing citral.

22
23 Solutions were made up of citral at 250, 125 and
24 62.5 ppm concentrations. Test solutions of these
25 three concentrations were prepared at different pHs
26 by adjusting the pH with HCl or NaOH to pH 4, 7 and
27 10.

28
29 One batch of test solutions was used immediately and
30 another was left for 24 hours before use. The
31 method of administration to the nematodes and

1 counting the kill rate is the same as for previous
2 protocols.

3

4 The results are shown below in Tables 11 and 12.
5 The figures given are for the percentage of
6 nematodes found to be dead upon microscopic
7 examination and are the average of 2 replicates.

8

9 **Table 11** - Effect of fresh citral at three pH levels
10 on root-knot nematodes (% nematodes dead)

11

	250 ppm			125 ppm			62.5 ppm			Water
PH	4	7	10	4	7	10	4	7	10	
24 h	75	73	83	31	44	39	48	39	32	21
48 h	73	72	87	50	47	39	50	44	45	30

12

13 **Table 12** - Effect of one-day old citral at three pH
14 levels on root-knot nematodes (% nematodes dead)

15

	250 ppm			125 ppm			62.5 ppm			water
PH	4	7	10	4	7	10	4	7	10	
24 h	90	40	47	27	25	25	40	30	16	10
48 h	90	33	52	31	33	32	27	27	21	14

16

17 The results demonstrate that, in general, the test
18 solutions lose efficacy if left for one day before
19 use. However, it was observed that the citral
20 solutions at the low pH (i.e. 4) did not lose
21 efficacy to such an extent and, in fact the 250 ppm
22 sample actually increased in efficacy after being
23 left for a day. At all concentrations tested, the
24 low pH samples did not demonstrate nearly such a

1 significant a drop of efficacy after being left when
2 compared to the neutral and high pH counterparts.

3
4 This demonstrates that low pH of citral is
5 beneficial in terms of retaining the efficacy of
6 citral as a nematocide over time. The reasons for
7 this are unclear, but may be the result of
8 stabilising the citral and preventing degradation.

9
10 It is therefore clear that adjusting the pH of a
11 citral containing nematocidal composition to be acid
12 (i.e. a pH below 7) would be beneficial in terms of
13 prolonging its action.

14
15 Example 10 - Comparison of nematocidal activity High
16 Purity Citral (98% pure) with Low Purity Citral (80%
17 pure).

18
19 Citral is commercially available in 2 forms -
20 regular (98% pure) and technical (80% pure). The
21 following protocol was carried out to determine if
22 technical citral is a viable alternative to pure
23 citral.

24
25 Compositions of regular and technical citral at 250
26 and 125 ppm were produced in 1% Tween 80 and
27 incubated with root-knot nematodes in the same way
28 as previously described. Observations of the kill
29 rate (percentage dead) were made at 21 and 42 hours.

30
31 The results are shown below in Table 13 and are the
32 average of four replicates.

1 **Table 13** - average percentage dead

2

	Citral (98% pure)		Citral (80% pure)		1% Tween 80	Water
Ppm	250	125	250	125	-	-
21 h	87	23	89	29	14	7
42 h	87	22	96	27	17	18

3

4 The results indicate that both regular and technical
5 citral kill nematodes effectively at concentrations
6 of 250 ppm. Thus technical citral may be used as a
7 cheaper alternative to regular citral.

8

9 Example 11 - Nematicidal Effects of Citral in Soil

10

11 The following protocol was carried out to assess the
12 nematicidal properties of nematodes in soil.

13

14 Methodology: Nematodes used for the analysis
15 originated from commercial agricultural crop soils.
16 Species of nematode included root-knot and citrus.
17 Prior to commencement of each study the nematodes
18 were counted and evaluated for viability. In each
19 experiment soil samples were infected with only one
20 species of nematode. Three measured quantities of
21 soil (250g) were placed into large PVC plastic
22 containers.

23

24 Soil moisture was assessed by weighing a soil sample
25 and then drying the sample in a drying oven. Soil
26 moisture content was confirmed using a "Hydroscout"
27 instrument. In all cases the moisture content

1 measured by both methods was within the resolution
2 of the instruments. By determining the water
3 content of the soil it was possible to calculate the
4 volume of terpene solution which would be diluted
5 when mixed with the soil.

6
7 A series of citral dilutions in water were prepared
8 (500 ppm to 62.5 ppm) such that when they were added
9 to the soil samples, they would yield the required
10 ratios. These dilutions were by volume not the more
11 commonly used mass ratios. The reason for using
12 volume dilutions was simply one of convenience
13 enabling the use of a micropipette or cylinder to
14 measure the terpene. The mass ratio of the 'in
15 soil' and 'in water' solution could be simply
16 calculated by multiplying the ppm of terpene by it's
17 density (0.92 g/ml).

18
19 The terpene solution was added to each test tube
20 containing a weighed sample of nematode infected
21 soil. The terpene solution and soil were mixed by
22 inverting the test tube several times. The test
23 tubes containing the soil and terpene solution were
24 left to stand in racks in the laboratory for 48
25 hours-72 hours depending on the survival of the
26 untreated nematodes. In each experiment a control
27 group was treated with distilled water. The %
28 mortality (kill) rates in the treatment groups was
29 compared with the control population.

30

1 The nematodes were extracted by "Sieving & mist
2 extraction" (Ayoub, S.M. 1977) prior to being
3 counted.

4

5 Criteria for Evaluation: Nematode counts were
6 performed to determine the proportion of nematodes
7 which survived and were killed in each treatment
8 group.

9

10 **Table 14** - Pretreatment nematode counts

11

Sample ID	Root-Knot	Citrus
Mean nematode counts (N=8)	659.25	12,711.75

12

13 The results are shown below in Tables 15 and 16.

14

15 **Table 15** - Treatment of Root Knot nematodes with
16 terpene solution.

17

Terpene concentration	No of Replicates	Mean % killed
500ppm	8	67.10
250ppm	8	23.66
125ppm	8	4.34
62.5ppm	8	18.87
untreated	8	5.71

18

19

20

21

1 **Table 16** - Treatment of Citrus nematodes with
2 terpene solution
3

Terpene concentration	No of Replicates	Mean % killed
500ppm	8	95.53
250ppm	8	91.66
125ppm	8	46.29
62.5ppm	8	-2.84
untreated	8	13.7

4
5 The protocol was repeated, this time using only
6 citral at 500 ppm concentration. The results are
7 shown below on Table 17 to 19.
8

9 **Table 17** - Pretreatment nematode counts
10

Sample ID	Root-Knot	Citrus
Mean nematode counts (N=8)	1225.25	10755.5

11
12 **Table 18** - Treatment of Root-Knot nematodes with
13 terpene solution
14

Terpene concentration	N	Mean % killed
500ppm	10	99.6

1 **Table 19** - Treatment of Citrus nematodes with
2 terpene solution

3

Terpene concentration	N	Mean % killed
500ppm	10	99.9

4
5 The experiment was performed once again, this time
6 with the following changes:

- 7 - Dose range of 125ppm-750ppm was used.
8 - Glass tubes containing 150g of soil were
9 used as opposed to PVC tubes in previous
10 experiments.

11
12 The results are shown below in Table 20.

13
14 **Table 20** - Treatment of Root Knot nematodes with
15 terpene solution

16

Terpene concentration	N	Mean % killed
750ppm	8	99.42
500ppm	8	100
250ppm	8	97.37
125ppm	8	74.51

17
18 The results all show that terpenes are effective
19 nematicides in soil. This supports the data already
20 provided showing that terpenes are effective
21 nematicides in vitro. Concentrations of terpene as
22 low as 125 ppm demonstrate strong nematicidal

1 activity in soil, though concentrations of 250 ppm
2 and above showed more consistent high kill rates.

3

4 Example 12 - Demonstration of Terpene Loading into
5 Bakers Yeast Particles and Purified Yeast Glucan
6 Particles

7

8 The following protocol was performed to demonstrate
9 that terpenes would load into yeast cell walls and
10 other yeast glucan particles.

11

12 Emulsions of citral and L-carvone were prepared by
13 mixing 150 µl of the terpene with 100 µl of 10%
14 Tween 80 in water and 250 µl of water.

15

16 Baker's yeast particles (YP) or LevacanTM yeast
17 glucan particles (YGP), available from Savory
18 Systems International, Inc., Branchburg, NJ, were
19 mixed with water to form a 250 mg/ml suspension.

20

21 500 µl of the YP or YGP suspension and 250 µl of the
22 terpene emulsion were mixed together and incubated
23 overnight under constant agitation. 500 µl YP or
24 YGP suspension and 500 µl of water were used as a
25 control. The particles were then washed with water
26 until free from external emulsion. The particle
27 preparations were then frozen and lyophilised until
28 dry.

29

30 The particles were then rehydrated and examined
31 under light microscope. The results are shown in
32 Figs. 1 to 4.

1 Fig. 1 shows spherical structures with a dark area
2 at their centre, these are empty hollow glucan
3 particles. Figs 2 and 3 shows spherical structures
4 with a swollen appearance with a light coloured
5 interior, these are particles with terpene
6 encapsulated in the central cavity - citral in Fig.
7 2 and L-carvone in Fig. 3. In Figs. 2 and 3 small
8 blobs of free terpene can also be seen, e.g. at the
9 top of Fig. 2, just left of centre. Figure 4 shows
10 the terpene emulsion as small blebs of terpene
11 suspended in water.

12

13 Example 13 - Determination of maximal citral and L-
14 carvone loading levels in Baker's Yeast Particles
15 (YP)

16

17 The following protocol was performed to determine
18 the maximal amounts of terpenes that would load into
19 YP.

20

- 21 - L-carvone and citral emulsions were prepared by
- 22 sonicating 4.5 g of the terpene with 0.3 ml
- 23 water.
- 24 - 10% Tween-80 solution was prepared by sonicating
- 25 4.5 g Tween-80 in 40.5 mls water.
- 26 - YP suspension was prepared by mixing YP with
- 27 water to form 20 mg/ml suspension.
- 28 - Encapsulation reactions were set up as described
- 29 in Table 21.

30

31 Citral or L-carvone-water emulsion was mixed with YP
32 and Tween 80 surfactant overnight at room

1 temperature. Samples were centrifuged at 14,000 x g
 2 for 10 minutes and the appearance of free terpene
 3 floating on the aqueous layer was scored. The
 4 results are shown in the right hand column labelled
 5 free terpene of Table 21.

6
 7 The expression "free terpene" refers to the visible
 8 presence of terpene in the centrifuged reaction
 9 mixture. The absence of free terpene indicates
 10 complete absorption of the terpene by the particles.
 11 The highest volume of terpene absorbed by the
 12 particles, as evidenced by the absence of free
 13 terpene, was recorded as the maximal volume of
 14 absorbed terpene emulsion.

15

16 **Table 21**

<u>Tube</u>	<u>20 mg/ml</u>	<u>Terpene</u>	<u>Vol</u>	<u>10% Tween-</u>	<u>Free</u>
	<u>YP</u>	<u>Emulsion</u>		<u>80</u>	<u>Terpene</u>
	μ l		μ l	μ l	
1	500	-	-	500	-
2	500	L-carvone	0.5	500	-
3	500	L-carvone	1.65	500	-
4	500	L-carvone	5	495	-
5	500	L-carvone	16.5	483.5	-
6	500	L-carvone	50	450	+
7	500	L-carvone	165	335	+
8	500	L-carvone	500	-	+
9	500	Citral	0.5	500	-
10	500	Citral	1.65	500	-
11	500	Citral	5	495	-
12	500	Citral	16.5	483.5	+/-
13	500	Citral	50	450	+

14	500	Citral	165	335	+
15	500	Citral	500	-	+

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As can be seen from the results, YP is capable of absorbing and encapsulating at least 16.5 μ l of L-carvone terpene emulsion or at least 5 μ l of citral emulsion per 10 mg of YP.

Example 14 - Demonstration of improved terpene loading with surfactant and determination of optimal Tween-80:Terpene ratio

The following protocol was performed to demonstrate that the presence of surfactant improves terpene loading and to determine the minimum level of Tween-80 surfactant required for the YP terpene loading reaction.

- L-carvone and citral emulsions were prepared by sonicating 4.5 g of the terpene with 0.3 ml water.
- 10% Tween-80 solution was prepared by sonicating 4.5 g Tween-80 in 40.5 ml water.
- Baker's YP suspension was prepared by mixing YP with water to form 250 mg/ml suspension.

Loading reactions were set up as shown in Table 22 below.

Citral or L-carvone-water emulsion was mixed with YP with 0 - 10% v/v Tween 80 surfactant overnight at room temperature. Samples were centrifuged at

1 14,000 x g for 10 minutes and the appearance of free
 2 terpene floating on the aqueous layer was scored.
 3 The results are shown in the right hand column
 4 labelled free terpene of Table 22.

5
 6 The expression "free terpene" refers to the visible
 7 presence of terpene in the centrifuged reaction
 8 mixture. The absence of free terpene indicates
 9 complete absorption and encapsulation of the terpene
 10 by the YP. The highest volume of terpene absorbed
 11 by the YP, as evidenced by the absence of free
 12 terpene, was recorded as the maximal volume of
 13 absorbed terpene emulsion.

14
 15 **Table 22**

<u>Tube</u>	<u>250</u> <u>mg/ml YP</u>	<u>Terpene</u> <u>Emulsion</u>	<u>Vol</u>	<u>10% Tween-</u> <u>80</u>	<u>Water</u>	<u>Free</u> <u>Terpene</u>
	ml		μl	μl	μl	
1	500	-	-	-	500	-
2	500	L-carvone	150	0	350	S1
3	500	L-carvone	150	5	345	S1
4	500	L-carvone	150	10	340	S1
5	500	L-carvone	150	33	317	S1
6	500	L-carvone	150	100	250	-
7	500	L-carvone	150	200	150	-
8	500	L-carvone	150	350	-	-
9	500	L-carvone	400	0	100	++
10	500	L-carvone	400	5	95	++
11	500	L-carvone	400	10	90	++
12	500	L-carvone	400	33	77	++
13	500	L-carvone	400	100	-	+
14	500	L-carvone	400	20 μl 100%	30	+

15	500	Citral	113	0	387	+
16	500	Citral	113	5	382	+
17	500	Citral	113	10	377	+
18	500	Citral	113	33	354	Sl
19	500	Citral	113	100	287	Sl
20	500	Citral	113	200	187	-
21	500	Citral	113	350	37	-
22	500	Citral	250	0	250	++
23	500	Citral	250	5	245	++
24	500	Citral	250	10	240	++
25	500	Citral	250	33	217	+
26	500	Citral	250	100	150	+
27	500	Citral	250	20 μ l 100%	230	+

1 Sl = slight

2

3 As can be seen from the results a Tween-80
 4 concentration of 1% (i.e. 100 μ l of 10 % Tween-80 in
 5 1000 μ l of reaction mixture) is sufficient to allow
 6 complete uptake of the terpene in the above
 7 reaction. A 2% Tween-80 causes no improvement in
 8 results, whereas with a 0.33% concentration free
 9 terpene was observed. This indicates that:

- 10 a) Terpenes are absorbed into YP particles in the
 11 absence of a surfactant, but the presence of
 12 surfactant significantly increases terpene
 13 absorption.
- 14 b) A Tween-80 concentration of around 1% is optimum
 15 for YP loading as it ensures proper loading
 16 whilst maximising the terpene payload of the YP
 17 particles.

18

19

1 Example 15 - Determination of maximal terpene
2 loading and encapsulation at high Baker's Yeast
3 Particles (YP) levels

4
5 The following protocol was performed to determine
6 the maximal amounts of terpenes that would load into
7 YP at high YP levels.

- 8
9 - L-carvone and citral emulsions were prepared by
10 sonicating 4.5 g of the terpene with 3 ml 1%
11 Tween.
12 - 5% Tween-80 solution was prepared by sonicating
13 0.5 g Tween-80 in 9.5 ml water..
14 - YP suspension was prepared by mixing YP with
15 water to form 250 mg/ml suspension.
16 - Encapsulation reactions were set up as shown in
17 Table 23.

18
19 Citral or L-carvone-water emulsion was mixed with YP
20 and Tween 80 surfactant overnight at room
21 temperature. Samples were centrifuged at 14,000 x g
22 for 10 minutes and the appearance of free terpene
23 floating on the aqueous layer was scored. The
24 results are shown in the right hand column labelled
25 free terpene of Table 23.

26
27 The expression "free terpene" refers to the visible
28 presence of terpene in the centrifuged reaction
29 mixture. The absence of free terpene indicates
30 complete absorption of the terpene by the YP. The
31 highest volume of terpene absorbed by the YP, as
32 evidenced by the absence of free terpene, was

1 recorded as the maximal volume of absorbed terpene
2 emulsion.

3

4 **Table 23**

<u>Tube</u>	<u>250</u> <u>mg/ml YP</u>	<u>Terpene</u> <u>Emulsion</u>	<u>Vol</u>	<u>1% Tween-</u> <u>80</u>	<u>Free</u> <u>Terpene</u>
	μ l		μ l	μ l	
1	500	-	-	500	-
2	500	L-carvone	15	485	-
3	500	L-carvone	37.5	462.5	-
4	500	L-carvone	75	425	-
5	500	L-carvone	112.5	387.5	-
6	500	L-carvone	150	350	S1 +
7	500	L-carvone	225	275	+
8	500	L-carvone	450	50	+
9	500	Citral	15	485	-
10	500	Citral	37.5	462.5	-
11	500	Citral	75	425	-
12	500	Citral	112.5	387.5	S1 +
13	500	Citral	150	350	+
14	500	Citral	225	275	+
15	500	Citral	450	50	+

5

6 As can be seen from the results in Table 9, YP is
7 capable of absorbing and encapsulating terpenes at
8 high YP concentration. YP absorbed and encapsulated
9 at least 112.5 μ l of L-carvone terpene emulsion or
10 at least 75 μ l of citral emulsion per 125 mg of YP.
11 This demonstrates that the terpene encapsulation
12 reaction is independent of YP concentration within
13 the ranges tested.

14

1 Example 16 - Screen commercially available particles
 2 for terpene absorption

3
 4 The following protocol was performed to analyse the
 5 loading properties of different types of particles.
 6 The particles studied were Baker's Yeast Particles
 7 (Sigma Chemical Corp., St. Louis, MO), NutrexTM
 8 Walls (Sensient Technologies, Milwaukee, WI), SAF-
 9 MannanTM (SAF Agri, Minneapolis, MN), Nutricept
 10 WallsTM (Nutraceuticals Inc., Burnsville, MN), LevacanTM
 11 (Savory Systems International, Inc., Branchburg, NJ)
 12 and WGPTM (Alpha-beta Technology, Inc. Worcester,
 13 MA).

14
 15 L-carvone and citral emulsions were prepared by
 16 sonicating 7 g terpene + 3 ml 3.3% Tween-80.

17
 18 Table 24 below compares the purity with the number
 19 of yeast particles per mg and the packed solids
 20 weight/volume ratio.

21
 22 **Table 24**

<u>Yeast Particle</u>	<u>Purity</u> <u>% Beta 1,3-</u> <u>glucan</u>	<u>No. particles/mg</u>	<u>Mg particles/ml</u>
Bakers	11.2	4×10^7	250
Nutrex	24.5	1.7×10^8	58.8
SAF Mannan	33.4	2.4×10^8	41.7
		2.7×10^8	
Nutraceuticals	55.7	5.2×10^8	37
Levacan	74.6	1×10^8	19.2
WGP	82.1	3.5×10^8	10

1 From Table 24 it can be concluded that the number of
2 particles per mg is inversely proportional to
3 purity. Thus the number of particles per mg of WGP
4 is almost 10-fold higher than Baker's YP.

5

6 The YP suspensions were prepared as follows:

7

- 8 - Baker's yeast particle suspension (YP) was
9 prepared by mixing 250 mg YP / ml 1% Tween 80.
- 10 - Nutrex suspension was prepared by mixing 163 mg
11 Nutrex YGP / ml 1% Tween 80.
- 12 - SAF Mannan suspension was prepared by mixing 234
13 mg Biospringer YGP / ml 1% Tween 80.
- 14 - Nutricepts suspension was prepared by mixing 99
15 mg Nutricepts YGP / ml 1% Tween 80.
- 16 - Levacan suspension was prepared by mixing 217 mg
17 Lev YGP / ml 1% Tween 80.
- 18 - WGP suspension was prepared by mixing 121 mg WGP
19 YGP / ml 1% Tween 80.

20

21 The packed volume of the above particles is
22 identical which means that equal numbers of
23 particles were assayed.

24

25 Loading reactions were set up as shown in Table 25
26 and left to incubate overnight. Samples were
27 centrifuged at 14,000 x g for 10 minutes and the
28 appearance of free terpene floating on the aqueous
29 layer and the color of the encapsulated terpenes in
30 the pellet was scored. The results are shown in the
31 two right hand columns of Table 25. The highest
32 volume of terpene absorbed by particles as evidenced

1 by the absence of free terpene was recorded as the
2 volume of absorbed terpene emulsion.
3

Table 25

Tube	Particle	conc mg/ml	μ l	Terpene Emulsion	Vol μ l	1% Tween 80 μ l	Free Terpene	Colour
1	Baker's	250	500	L-carvone	125	375	-	W
2	Nutrex	163	500	L-carvone	125	375	-	W
3	SAF Mannan	234	500	L-carvone	125	375	-	W
4	Nutricepts	99	500	L-carvone	125	375	+	W
5	Levacan	217	500	L-carvone	125	375	+	W
6	WGP	121	500	L-carvone	125	375	+	W
7	Baker's	250	500	Citral	100	375	-	Y
8	Nutrex	163	500	Citral	100	375	-	Y
9	SAF Mannan	234	500	Citral	100	375	-	W
10	Nutricepts	99	500	Citral	100	375	+	Y
11	Levacan	217	500	Citral	100	375	+	int
12	WGP	121	500	Citral	100	375	+	int
13	-	-	-	L-carvone	125	875	+	-
14	-	-	-	Citral	100	900	+	Y

W = white; Y = yellow; sl = slight; int = intermediate

1 From the results the following conclusions were
2 reached:

- 3 - Purified particles with a low lipid content were
4 less effective at absorbing terpenes.
- 5 - Less pure particles were more effective at
6 absorbing terpenes.
- 7 - Yellow degradation product of citral was not
8 formed when encapsulated in SAF-MannanTM.
- 9 - Based on qualitative loading at the single
10 terpene level tested, SAF MannanTM appears to be
11 best, NutrexTM second and Baker's third.

12
13 Example 17 - Kinetics of terpene loading into
14 various types of particles and different incubation
15 temperatures.

16
17 The following protocol was adopted to compare the
18 loading kinetics of various types of yeast
19 particles.

20
21 L-carvone and citral emulsions were prepared by
22 sonicating 7 g terpene with 3 ml 3.3% Tween-80.

23
24 1% Tween-80 solution was prepared by sonicating 1 ml
25 10% Tween-80 in 10 ml water.

26
27 - Baker's YP was prepared by mixing 5 g of bakers
28 YP in 20 ml 1% Tween-80.

29 - NutrexTM YGP suspension was prepared by mixing 2
30 g NutrexTM YGP in 20 ml 1% Tween-80.

31 - SAF MannanTM suspension was prepared by mixing 2
32 g SAF MannanTM in 20 ml 1% Tween-80.

1 Loading reactions were set up as shown in Table 26.

2

3 The reactions were incubated for 1, 3, 6, 9 and 24
4 hours at room temperature or 37 °C. After
5 incubation samples were centrifuged at 14,000 x g
6 for 10 minutes and the appearance of free terpene
7 floating on the aqueous layer was scored. The
8 results are shown in the two right hand columns of
9 Table 26. The highest volume of terpene absorbed by
10 the particles as evidenced by the absence of free
11 terpene was recorded as the volume of absorbed
12 terpene emulsion. Colour of the encapsulated pellet
13 was scored at 24 hours.

14

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1 Table 26

Tube	T °C	Particle	conc mg/ml	µl	Terpene Emulsion	Vol µl	1% Tween-80	Free Terpene (hr)					Color
								1	3	6	9	24	
1	Rt	Bakers	250	3500	L-carvone	788	2712	+	-	-	-	-	W
2	37	Bakers	250	3500	L-carvone	788	2712	+	-	-	-	-	W
3	Rt	Nutrex	100	3500	L-carvone	1050	2450	+	-	-	-	-	W
4	37	Nutrex	100	3500	L-carvone	1050	2450	+	-	-	-	-	W
5	Rt	SAF	100	3500	L-carvone	1050	2450	<+	-	-	-	-	W
6	37	SAF	100	3500	L-carvone	1050	2450	<+	-	-	-	-	W
7	Rt	Bakers	250	3500	Citral	525	2975	+	-	-	-	-	Y
8	37	Bakers	250	3500	Citral	525	2975	+	-	-	-	-	VY
9	Rt	Nutrex	100	3500	Citral	788	2712	+	-	-	-	-	Y
10	37	Nutrex	100	3500	Citral	788	2712	+	-	-	-	-	VY
11	Rt	SAF	100	3500	Citral	788	2712	+	-	-	-	-	W
12	37	SAF	100	3500	Citral	788	2712	+	-	-	-	-	W

White, W; Yellow, Y; Very Yellow, VY; Room Temperature, Rt

1 From the results shown in Table 26 and other ,
2 observations the following conclusions can be made:

- 3 • Terpene loading reaction takes between 1 and 3
4 hours.
- 5 • Terpene loading occurs faster at 37 °C than at
6 room temperature.
- 7 • SAF MannanTM appears to be preferable particles
8 for two reasons:
 - 9 - Faster and more complete uptake of both
10 terpenes.
 - 11 - Citral remains stable when loaded as
12 evidenced by the absence of yellow colour,
13 characteristic of citral degradation, after
14 24 hours at 37 °C.

15
16 Example 18 - Screen a range of single terpenes and
17 terpene combinations for particle loading
18

19 The following protocol was adopted to compare the
20 loading efficiency of Baker's YP versus SAF MannanTM.
21

22 Terpene emulsions were prepared as follows:

- 23 - L-carvone - 4.5 g L-carvone in 1.5 ml 3.3% Tween-
24 80.
- 25 - Citral - 4.5 g citral in 1.5 ml 3.3% Tween-80.
- 26 - Thymol/L-carvone mixture (T/L)- 2.25 g thymol and
27 2.25 g L-carvone in 1.5 ml 3.3% Tween-80.
- 28 - Eugenol - 4.5 g eugenol in 1.5 ml 3.3% Tween-80.
- 29 - Geraniol - 4.5 g geraniol in 1.5 ml 3.3% Tween-
30 80.

1 - Citral/L-carvone/Eugenol mixture (C/L/E) - 1.5 g
2 citral, 1.5 g L-carvone, 1.5 g eugenol in in 1.5
3 ml 3.3% Tween-80.

4

5 Emulsions composed of terpene : water : surfactant
6 ratio of 0.75:0.3:0.05 were used for these
7 experiments.

8

9 Increasing volumes of terpene emulsion were mixed
10 with 250 mg/ml Baker's YP or 250 mg/ml SAF MannanTM
11 overnight at room temperature as shown in Tables 27
12 and 28. Samples were centrifuged at 14,000 x g for
13 10 minutes and the appearance of free terpene
14 floating on the aqueous layer was scored. The
15 highest volume of terpene emulsion absorbed by
16 Baker's YP or SAF MannanTM as evidenced by the
17 absence of free terpene was recorded as the volume
18 of absorbed terpene emulsion. Colour of encapsulated
19 terpenes in the pellet was recorded. The results in
20 Tables 27 and 28 show that all single and terpene
21 combinations were efficiently loaded into both
22 Baker's YP or SAF Mannan particles.

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- 1 Table 27 - Evaluation of Baker's YP Loading of
 2 Different Terpenes and Terpene Mixtures.

<u>Tube</u>	<u>Baker</u> <u>(μl)</u>	<u>Terpene</u> <u>Emulsion</u>	<u>Vol</u> <u>(μl)</u>	<u>1% Tween-</u> <u>80 (μl)</u>	<u>Free</u> <u>Terpene</u>	<u>Colour</u>
1	500	-	-	500	-	W
2	500	L-carvone	15	485	-	W
3	500	L-carvone	37.5	462.5	-	W
4	500	L-carvone	7	425	+/-	W
5	500	L-carvone	112.5	387.5	+/-	W
6	500	L-carvone	150	350	+	W
7	500	L-carvone	225	275	+	W
8	500	L-carvone	450	50	++	W
9	500	Citral	15	485	-	Y
10	500	Citral	37.5	462.5	-	Y
11	500	Citral	75	425	-	Y
12	500	Citral	112.5	387.5	+/-	Y
13	500	Citral	150	350	+	Y
14	500	Citral	225	275	+	Y
15	500	Citral	450	50	+	Y
16	500	T/L	15	485	-	W
17	500	T/L	37.5	462.5	-	W
18	500	T/L	75	425	-	W
19	500	T/L	112.5	387.5	+/-	W
20	500	T/L	150	350	+	W
21	500	T/L	225	275	+	W
22	500	T/L	450	50	+	W
23	500	Eugenol	15	485	-	W
24	500	Eugenol	37.5	462.5	-	W
25	500	Eugenol	75	425	-	W
26	500	Eugenol	112.5	387.5	+/-	W
27	500	Eugenol	150	350	+	W

28	500	Eugenol	225	275	+	W
29	500	Eugenol	450	50	+	W
30	500	Geraniol	15	485	-	W
31	500	Geraniol	37.5	462.5	-	W
32	500	Geraniol	75	425	-	W
33	500	Geraniol	112.5	387.5	+	W
34	500	Geraniol	150	350	+	W
35	500	Geraniol	225	275	+	W
36	500	Geraniol	450	50	+	W
37	500	C/L/E	15	485	-	Y
38	500	C/L/E	37.5	462.5	-	Y
39	500	C/L/E	75	425	-	Y
40	500	C/L/E	112.5	387.5	+/-	Y
41	500	C/L/E	150	350	+	Y
42	500	C/L/E	225	275	+	Y
43	500	C/L/E	450	50	+	Y

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- 1 Table 28 - Evaluation of SAF Mannan Loading of
 2 Different Terpenes and Terpene Mixtures.
 3

<u>Tube</u>	<u>SAF</u> <u>(μl)</u>	<u>Terpene</u> <u>Emulsion</u>	<u>Vol</u>	<u>1% Tween-</u> <u>80 (μl)</u>	<u>Free</u> <u>Terpene</u>	<u>Colour</u>
1	500	-	-	500	-	W
2	500	L-carvone	15	485	-	W
3	500	L-carvone	37.5	462.5	-	W
4	500	L-carvone	75	425	-	W
5	500	L-carvone	112.5	387.5	-	W
6	500	L-carvone	150	350	+/-	W
7	500	L-carvone	225	275	+/-	W
8	500	L-carvone	450	50	+	W
9	500	Citral	15	485	-	W
10	500	Citral	37.5	462.5	-	W
11	500	Citral	75 μ l	425	-	W
12	500	Citral	112.5	387.5	-	W
13	500	Citral	150	350	+/- Inverted	W
14	500	Citral	225	275	+ Inverted	W
15	500	Citral	450	50	+ Inverted	W
16	500	T/L	15	485	-	W
17	500	T/L	37.5	462.5	-	W
18	500	T/L	75	425	-	W
19	500	T/L	112.5	387.5	-	W
20	500	T/L	150	350	+/-	W
21	500	T/L	225	275	+	W
22	500	T/L	450	50	+	W
23	500	Eugenol	15	485	-	W
24	500	Eugenol	37.5	462.5	-	W
25	500	Eugenol	75	425	-	W

26	500	Eugenol	112.5	387.5	+/-	W
27	500	Eugenol	150	350	+	W
28	500	Eugenol	225	275	+	W
29	500	Eugenol	450	50	+	W
30	500	Geraniol	15	485	-	W
31	500	Geraniol	37.5	462.5	-	W
32	500	Geraniol	75	425	-	W
33	500	Geraniol	112.5	387.5	-	W
34	500	Geraniol	150	350	-	W
35	500	Geraniol	225	275	- Inverted	W
36	500	Geraniol	450	50	+ Inverted	W
37	500	C/L/E	15	485	-	W
38	500	C/L/E	37.5	462.5	-	W
39	500	C/L/E	75	425	-	W
40	500	C/L/E	112.5	387.5	-	W
41	500	C/L/E	150	350	-	W
42	500	C/L/E	225	275	+/-	W
43	500	C/L/E	450	50	+	W

1

2

Inverted = Phase Inverted - solids floating on top

3

- no free oil; W = white; Y = yellow.

4

5

From the results the following observations were

6

made:

7

- All terpenes appeared to load into Baker's YP and
SAF Mannan.

8

9

- SAF Mannan has a higher terpene loading capacity
than bakers YP.

10

11

- The two and three way mixtures of terpenes also

12

appear to efficiently load.

- 1 - The terpene Eugenol appears to have a higher
2 density than the particles and water as it was
3 found associated with the pellet.
- 4 - For the SAF Mannan, the higher load levels and
5 lighter particles resulted in loaded particles
6 floating on the surface of the aqueous layer for
7 citral and geraniol.
- 8 - Citral was protected from oxidation by the SAF
9 Mannan but not by the Baker's YP.

10

11 The approximate maximal loading for each particle
12 type was determined and is shown in tables 29 and 30
13 below. Percentage loaded represents a ratio of the
14 amount of terpene loaded to the amount of particle
15 present (weight for weight).

16

17 Table 29 - Maximal terpene loading in Baker's YP.

18

<u>Terpene</u>	<u>Vol. Loaded μl</u>	<u>% Loaded w/w</u>
L-carvone	37.5	33.3
Citral	75	67%
Thymol/L-carvone 1:1	75	67%
Eugenol	75	67%
Geraniol	75	67%
Citral/L-carvone/ Eugenol (1:1:1)	75	67%

19

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24

1 Table 30 - Maximal terpene loading in SAF Mannan.

2

<u>Terpene</u>	<u>Vol. loaded μl</u>	<u>% Loaded w/w</u>
L-carvone	112.5	100%
Citral	150	133%
Thymol/L-carvone 1:1	112.5	100%
Eugenol	112.5	100%
Geraniol	150	133%
Citral/L-carvone/ Eugenol (1:1:1)	150	133%

3

4 Example 19 - Evaluation of Terpene stability in
5 aqueous emulsions and encapsulated terpene
6 formulations

7

8 Terpene stability was assessed by the observation of
9 citral formulations for the formation of a yellow
10 colored oxidation product. As noted in the right
11 hand column in Tables 25 - 28 citral emulsions and
12 citral encapsulated Bakers YP turned a progressively
13 increasing yellow color over time. However, citral
14 encapsulation in SAF MannanTM increased citral
15 stability as evidenced by a reduction or absence of
16 yellow color over time.

17

18 Example 20 - Loading of Terpenes in minimal water

19

20 The following protocol was carried out to evaluate
21 the possibility that terpene loading and
22 encapsulation into YP could be carried out at a very
23 high Yeast Particles (YP) solids level to allow for
24 direct extrusion of the loaded formulation into a

1 fluidised bed drier. The minimal amount of water to
2 completely hydrate the SAF MannanTM particles was
3 determined to be 3.53 g water per g solids. This
4 defines the hydrodynamic volume (HV) or water
5 absorptive capacity of the particles. At this level
6 of water the hydrated particles have a consistency
7 of a stiff dough which is thixotropic, i.e. shear
8 thinning like mayonnaise. Addition of water up to
9 40 % above the HV results in a thick flowable paste.
10 The standard reaction that has been used in the
11 above examples was carried out at 3 X HV water.

12
13 A series of terpene (L-carvone) loading reactions
14 were carried out keeping the ratio of
15 particle:terpene:Tween (1: 0.44:0.04) constant and
16 varying the amount of water in the system from the
17 HV (3.53 g) to HV + 40% water (4.92 g). Controls
18 were the standard loading system which uses 3 X HV
19 water, particles only and terpene only reactions.
20 Following overnight incubation samples of the
21 mixtures were evaluated microscopically for free
22 terpene and evidence of terpene uptake into the
23 particles and for material flow characteristics by
24 assessing flow in inverted tubes over 15 minutes.
25 In addition, the presence of free oil was assessed
26 by hydrating the reaction mixture with 5 X HV,
27 vortexing to obtain a complete dispersion of
28 particles and centrifugation to sediment the
29 particle encapsulated terpene. The results are
30 shown in Table 31 and Figs. 7 to 12. Figs. 7 to 12
31 show the loading results of the following tubes:

32

- 1 - Fig. 7 - Tube 3
 2 - Fig. 8 - Tube 5
 3 - Fig. 9 - Tube 6
 4 - Fig. 10 - Tube 8
 5 - Fig. 11 - Tube 10
 6 - Fig. 12 - Tube 11

7

8 Table 31

<u>Tube</u>	<u>SAF</u> <u>g</u>	<u>Terpene</u> <u>Emulsion</u>	<u>Weight</u> <u>(g)</u>	<u>Water</u> <u>(g)</u>	<u>Free</u> <u>Terpene</u>	<u>Flow</u>
1	-	L-carvone	4.64	4.5	+	+
2	1	-	-	8.0	-	+
3	1	L-carvone	4.64	4.5	-	+
4	1	L-carvone	4.64	-	-	-
5	1	L-carvone	4.64	0.17	-	-
6	1	L-carvone	4.64	0.35	-	-
7	1	L-carvone	4.64	0.52	-	Sl
8	1	L-carvone	4.64	0.7	-	Mod
9	1	L-carvone	4.64	0.87	-	High
10	1	L-carvone	4.64	1.05	-	High
11	1	L-carvone	4.64	1.39	-	High

9

10 The results shown in Table 31 and Figs. 7 to 12
 11 demonstrate that terpene loading and encapsulation
 12 into the particles occurred at all water ratios
 13 evaluated. Surprisingly, equivalent loading
 14 occurred even when the loading reaction was taking
 15 place in a reaction with the consistency of a stiff
 16 dough using the minimal amount of water to hydrate
 17 the particles. The absence of free terpene was
 18 observed microscopically (Figs. 7 to 12) and in the

1 low level of terpene in the supernatants, as ,
2 evidenced by a marked reduction in the turbidity of
3 the supernatant compared to the terpene only
4 control.

5
6 These results extend our understanding of the
7 conditions to load terpenes into hollow glucan
8 particles. The flexibility to use a minimal volume
9 of water to hydrate the particles during the loading
10 process will allow loading of the terpenes under
11 conditions where the reaction mixture is a malleable
12 dough-like consistency using standard food-grade
13 swept surface dough mixers. The consistency of the
14 final high solids terpene loaded mixture is suitable
15 for direct extrusion to form noodles and pellets for
16 fluidised bed drying.

17
18 Suitable facilities to scale up production in this
19 manner would require:

- 20 - Gaulin homogeniser, or equivalent to produce
- 21 stable terpene emulsion.
- 22 - Swept surface dough mixing tank.
- 23 - Extruder.
- 24 - Fluidised bed drier.

25
26 Example 21 - Evaluation of an interstitial
27 hydrocolloid agent to aid dispersion in dried hollow
28 glucan particles encapsulating a terpene component
29 dispersion when re-hydrated.

30
31 The following protocol was adopted to evaluate the
32 effect of an interstitial hydrocolloid to increase

1 dried hollow glucan particle encapsulated terpene
2 formulations to disperse when hydrated.

3

- 4 - SAF MannanTM particles
- 5 - 0.1% Tween 80
- 6 - L-carvone
- 7 - Xanthan Gum - 1% w/v in 0.1% Tween 80

8

9 The effect of increasing xanthan gum levels on dry
10 hollow glucan particle encapsulated L-carvone
11 dispersion in water was assessed by loading L-
12 carvone into SAF Mannan by incubating 1.1 g of an L-
13 carvone emulsion (L-carvone : water : surfactant
14 ratio of 0.75:0.3:0.05) with 1 g SAF Mannan and 4.4
15 g 0.1% Tween 80 containing 0 - 1% xanthan gum as
16 shown in Table 32.

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1 Table 32

<u>Tube</u>	<u>SAF</u> <u>g</u>	<u>L-carvone</u> <u>Emulsion</u> <u>(g)</u>	<u>0.1%</u> <u>Tween-80</u> <u>(g)</u>	<u>1%</u> <u>Xanthan</u> <u>(g)</u>	<u>Visual</u> <u>Observations</u>
1	1	1.1	4.4	0	Large non-uniform clumps
2	1	1.1	4.33	0.07	Uniform suspension
3	1	1.1	4.26	0.14	Uniform suspension
4	1	1.1	4.12	0.28	Uniform suspension
5	1	1.1	3.85	0.55	Uniform suspension
6	1	1.1	3.3	1.1	Finer Uniform suspension
7	1	1.1	2.2	2.2	Finer Uniform suspension
8	1	1.1	0	4.4	Finer Uniform suspension

2
3
4 The results in Table 32 and Figs 13 to 20
5 demonstrate that the inclusion of a high molecular
6 weight hydrocolloid during the drying of the
7 particle encapsulated terpene aids in the hydration
8 and dispersion of the microparticles into a uniform
9 suspension. Other examples of such hydrocolloid
10 agents are maltodextrin, alginates, or the like.

11
12 It may also be worthwhile to include a pellet
13 coating to increase the stability of the loaded

1 terpenes, and to provide a sustained release of
2 terpene.

3
4 Example 22 - Nematocidal Activity of Encapsulated
5 Terpenes

6
7 Preparations of yeast cell walls encapsulating
8 citral were prepared according to the procedures
9 described above. The hollow glucan particles
10 contained 17.5% citral, and the particles were
11 present at in the test preparations at a
12 concentration of 1000 ppm. This means that terpenes
13 were effectively present at a concentration of 175
14 ppm.

15
16 1.0 ml of the test preparations was added to 0.1 to
17 0.15 ml of water containing root-knot nematodes.
18 1.0 water was added to the nematodes as the control.

19
20 Observations were made as [revopis;u descrobed and
21 the kill rate assessed (i.e. percentage dead) after
22 24 and 48 hrs. The results shown below in Table 13
23 are an average of 2 sets of results.

24
25 Table 33 - Nematicidal activity of encapsulated
26 terpene solution (17.5 % citral @ 1000ppm)

27

Time	Kill Rate	
	Test	Control
24 h	45	17
48 h	56	21

28

1 The results demonstrate that hollow glucan particles
2 encapsulating terpenes are effective at killing
3 root-knot nematodes at a particle concentration of
4 1000 ppm, which corresponds to a citral
5 concentration of only 175 ppm.

6
7 Thus hollow glucan particles encapsulating terpenes
8 appear to be as effective as terpenes in solution or
9 with surfactant as nematicides. The nematicidal
10 activity is retained despite the terpene being
11 encapsulated within the particle. It can be
12 expected that higher concentrations of terpenes
13 within the hollow glucan particles, or higher
14 concentrations of the particles would result in an
15 even higher kill rate, as is the case for terpenes
16 in solution or with surfactant.